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EMBRYOLOGICAL DEVELOPMENT OF THE POLYCHAETOUS ANNELID, DIOPATRA CUPREA (BOSC)[1]

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Insofar as the writer knows, the normal embryology of *Diopatra cuprea* has never been completely worked out. The main trouble seems to have been that investigators, with the exception of Just (1922), have found that it is difficult to activate the eggs of this species even when they appear ripe. Andrews had similar difficulty with the eggs of the closely related species, *Diopatra magna* (since designated *Onuphis magna*). He made the statement (1891b, page 115) that "attempts at artificial fertilization were unsuccessful" although the eggs seemed ripe as indicated by their size and the large numbers present packing the coelom, as well as the occasional finding of similar eggs amongst the larvae in the egg masses which he found during the breeding season. However, Just (1922), in a paper concerned primarily with raising mature *Platynereis megalops* from eggs, noted (page 477), "Though it is usually stated that artificial insemination of Diopatra eggs is not possible, every attempt made by the writer . . . was successful," and that he reared *Diopatra cuprea* to a length of 4 cm. No record of development was given.

The problem of activation has remained a significant one throughout the course of this investigation. With perseverance (particularly initially) larvae from many batches of eggs have been raised during the course of several summers to a stage where 6 sets of setae have been formed and, in the summer of 1958, a few were raised to a stage with 7 sets of setae. Thus far two abstracts have been published on this work (Allen, 1951, 1953) and more recently Costello *et al.* (1957) have included some additional previously unpublished data (furnished by the present writer) in their book on handling marine eggs and embryos.

The study of the development of *D. cuprea* is still incomplete but enough additional material has recently been worked out so that it was thought advisable to publish a more detailed account of development than has thus far been done. There is little material in the literature on the development of the genus, Diopatra. As further observations on living material were made, the confusion in the literature surrounding the development of the species, *D. cuprea*, became more apparent.

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Observations made during the present study suggest that most, if not all, of the material which has been published on the development of *Diopatra cuprea* has been incorrectly attributed to this species, so that the investigations of the writer may represent the only material published on the development of this polychaete.

Material and Methods

The adult worm. The characteristics and habits of the adult worms of this species have been described by various investigators (Andrews, 1891a; Sumner, Osborn and Cole, 1911; Hartman, 1945, 1951; *et al.*). The parchment-like tubes of these polychaetes, which are found in the intertidal zone, go down two to three feet into the substratum. When disturbed the worm retreats into the tube so that in digging for the adults one rarely obtains the whole worm. As a result, the posterior tip with its four anal cirri is rarely seen. The head bears five occipital tentacles and two shorter frontal tentacles. Larvae have been raised to a stage when the five occipital tentacles and two anal cirri are noticeable. Males which are sexually mature are cream to yellowish in color as a result of the sperm packed in the coelom. In males with fewer sperm only the parapodia are yellowish in color. Females when sexually mature are usually grey-green due to the color of the eggs (which have a green pigment) packed in the coelom. This species is plentiful in Woods Hole waters. Most of the collecting for this investigation was done at Northwest Gutter, Hadley Harbor, Massachusetts, and some of it was done in the harbor at North Falmouth and at Woods Hole, on the Buzzards Bay side. The adults for the most part were kept in aquaria in running sea water. The worms were fed every day or two with pieces of the mussel, Mytilus.

Procuring and handling living developmental stages. The writer has raised larvae of *Diopatra cuprea* from mid-June through August following artificial fertilization. The problem of activation of apparently ripe eggs was present throughout this period but artificial insemination was more successful in June and July than in August. This is contrary to the remark of Bumpus (1898, page 855) that "the ova are nearly ripe in August."

During the breeding period of a sexually mature worm, the coelom becomes packed with gametes. When eggs or sperm are needed, the posterior end of a worm is exposed by cutting the end of the tube with scissors. The exposed portion is then held lightly with forceps. This usually results in the worm's pinching off its posterior segments. Eggs were obtained from the isolated posterior sections by slitting the body wall along the bases of the parapodia with No. 5 watchmaker's forceps. Eggs thus obtained were washed in Syracuse dishes with sand-filtered sea water. In general, spermatozoa were obtained by making a small slit at the base of a parapodium with a No. 5 watchmaker's forceps and diluting the "dry" sperm with sand-filtered sea water. Under the dissecting microscope ripe sperm were observed to be active immediately. Polyspermy should be avoided.

Within a few minutes after insemination the eggs were washed several times with sand-filtered sea water. Usually they were given fresh sea water one to two hours later. If development were normal, ciliated larvae developed at room temperature within three hours after insemination. At this stage larvae usually were transferred to stender dishes and placed on the sea water table in a moist

chamber with 90% sea water in the bottom. The water was changed at least once a day thereafter.

Apparently egg laying in *D. cuprea* is a phenomenon rarely observed (Sumner *et al.*, 1911). In only one instance did the writer observe natural egg laying in the laboratory. This was on the evening of June 23, 1949. A worm tube was picked up and eggs were immediately released in a transparent, only slightly viscous, jelly which dissolved readily in sea water. The eggs were fertilized artificially and almost 100% cleaved. Only a few other times in the experience of the writer has fertilization approached 100%, as the method of artificial insemination described is frequently unsuccessful. To get a batch of eggs with 50% of the eggs cleaving is good.

Observations were made on living stages with the dissecting and compound microscopes and, in the summer of 1958, additional observations were made with the phase microscope. For study and for photomicrographs the ciliated stages were slowed down with a little dry MS-222 (tricain) added with a dissecting needle to a drop of filtered sea water containing the larvae (optimal concentrations for quieting various larval stages were not determined).

For the setal studies the larvae were placed on a slide in a drop of filtered sea water and then a cover slip was applied. They were examined briefly under a magnification of × 430 and then left to dry a little. This treatment in many cases spread out the setae which were then studied in more detail under × 430.

Handling of fixed material. Various stages were fixed, paraffin-embedded, and serially sectioned (usually at 7 or 10 micra). Whole mounts of stained and unstained stages were also made. The fixatives used for the early stages were usually Allen's B-15 or Bouin's, and for later larval stages Schaudinn's or Bouin's heated to 60° C. A series was also fixed in Meves'. A variety of stains was tried including Heidenhain's hematoxylin, Harris' hematoxylin, acetocarmine, alum-cochineal, Giemsa's, toluidin blue, and Feulgen's. Sections and whole mounts usually were mounted in Permount or Canada balsam. It was considered important to use such whole mounts to make a cell lineage study through at least the early cleavage stages. However, the method which had given excellent results with cleavage stages of the gastropod, Crepidula, failed completely with Diopatra. Various other techniques have been tried, including pre-treatment to remove lipids or ribonucleic acid, either of which might take up the stain in the cytoplasm. To date a technique has not been developed that would stain the chromosomes and enable one to follow the orientation of the spindles without staining the cytoplasm.

Normal Development

The writer has indicated already (1951) that the cleavage of *Diopatra cuprea* occurs with amazing rapidity, functional cilia being formed within three hours after insemination. Prior to this age, it is difficult to construct a time table of development because there is considerable variability among different batches of eggs and also among different eggs in the same batch, particularly in cases in which low percentages of fertilization occur. The following represents a slight elaboration of the schedule recorded in Costello *et al.* (1957) which is based on the writer's data obtained over several summers. The times are recorded from insemination at temperatures of 21–24° C.

Stage	Time
First polar body	15–20 minutes
Second polar body	20–30 minutes
Two- to four-cells	40–60 minutes
Eight-cells	50–90 minutes
Mid- to late cleavage	90–120 minutes
Functional cilia	3 hours
Apical tuft (apparent in some)	8–9 hours
Apical tuft (present in all normal larvae)	12 hours
Rotating trochophores	24 hours
2 to 3 sets of internal setae	36 hours
3 sets of external setae, no tentacles	2 days
4 sets of external setae, some with 3 tentacles	3–4 days
5 sets of external setae, 5 tentacles	$4\frac{1}{2}$–$5\frac{1}{2}$ days
6 sets of external setae, 5 tentacles	$6\frac{1}{2}$–8 days
7 sets of external setae, 5 tentacles	13–17 days (typical?)

The various stages of normal development are described in more detail below.

The unfertilized egg. In *Diopatra cuprea* the unfertilized egg is oval. After its growth period the average size of the egg is approximately 235–240 × 205–210 micra (Fig. 2). (Andrews, 1891b, gives the diameter of this egg as 400 micra; however, the above dimensions are based on repeated measurements by the writer.) In living eggs the germinal vesicle is visible as a lighter region near the animal pole. Surrounding it is an area of non-yolky cytoplasm in which are suspended bright green pigment granules. External to these are yolk granules which increase in number towards the vegetal pole. Their accumulation thus establishes a visible animal-vegetal gradient and makes the egg very opaque. In reflected light under the dissecting microscope the eggs *en masse* in some batches are creamy yellow or creamy white; in other batches, eggs have a greenish hue. With dark-field (under low power) the eggs are a rich yellowish cream color with brilliant green granules obvious around the germinal vesicle. The differences in color apparent under the dissecting microscope are due to the relative amounts of green pigment and yolk. The egg has a clearly defined membrane, approximately 3 micra thick, which appears to be perforated by radial pores when viewed under the compound microscope.

A curious feature of the development of these eggs is the two strings of cells attached to them during their growth period in the coelom. Andrews (1891b) described these follicle cells and states (page 113) that "these objects were at first mistaken for parasitic algae." These "nurse" cells are transparent (Fig. 1) and bear a striking resemblance to blue-green algae. However, each algal-like cell has a relatively large nucleus with a prominent nucleolus (Fig. 16). In the very small oocytes bearing these "nurse" or follicle cells, the pigment is a brilliant green as there is little or no yolk to mask it. These smaller eggs have a central nucleus (Fig. 16). Subsequently, with the differential accumulation of yolk, the nucleus becomes excentrically located, coming to lie near the animal pole (region indicated in Figure 17).

Apparently the follicle cells are not lost until the end of the growth period in oogenesis as a few full-size eggs have been observed with these algal-like strings attached. Andrews (1891b) has observed that these cell strings are retained in *D. magna* until near the end of the growth period.

Fertilization. The egg is fertilizable at the germinal vesicle stage. The first indication of fertilization is the lifting off of the egg membrane to form the fertiliza-

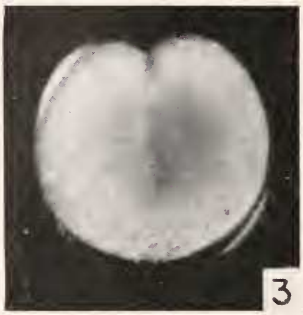
tion membrane. The perivitelline space is slight, being most obvious in the region of the animal pole. The germinal vesicle becomes less and less distinct as the perivitelline space forms. Usually the first polar body is given off within 20 minutes after insemination and the second within 30 minutes after insemination. A small pigment-free area around the animal pole marks the position where the second polar body will pinch off (Fig. 2). The polar bodies are small (Fig. 4), the second polar body being somewhat larger than the first. Figure 17 shows a section of an egg in metaphase I (the polar bodies are not visible).

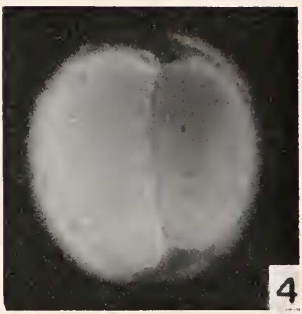
Cleavage. Occasionally the two-cell stage may be observed 30 minutes after insemination but usually the first cleavage is not completed until 40 minutes or so after insemination. The first cleavage furrow is meridional, cutting through the animal pole first (Fig. 3) resulting in two blastomeres of unequal size, the AB being somewhat smaller than the CD blastomere (Figs. 4 and 18). There is some variation in the size difference between the first two blastomeres. Blastomeres AB and CD often divide about the same time as seen in living stages and sections. In some cases the larger blastomere appears to divide first, as three-cell stages may be observed (Fig. 19). It is possible, however, that these three-cell stages represent abnormal development. The four-cell stage shows a cross furrow with the arrangement of cells typical of spiral cleavage (Fig. 5). In living stages the nuclei appear as lighter regions. The third cleavage results in an eight-cell stage with four somewhat smaller micromeres being polar in position (Fig. 6). Cleavages beyond these first few are amazingly rapid. A mid-cleavage stage is shown in Figure 7. During late cleavage the blastomeres are held firmly together within the original egg membrane, and a vacuolated peripheral area is appearing (Fig. 20).

Early ciliated stages (3 to 12 hours). These stages are approximately the size of the unfertilized egg. While there is no increase in mass, cells are continuing to divide. Gastrulation in these very rapidly developing early stages may be occurring by the time cilia have differentiated and probably takes place primarily by epiboly.

Functional cilia penetrate the egg membrane within three hours after insemination. They appear to push through the pores noted above in the membrane of the unfertilized egg. It is difficult to be certain of the ciliary distribution even when using the phase contrast microscope, but cilia appear to cover the entire surface except for two areas, a disc at the posterior end and the region around the future apical tuft. The cilia thus appear to form a very broad band involving most of the larva. At this stage peripheral vacuolated cells form four anterior plates which surround, and appear distinct from, a central mound of denser cells (Figs. 8, 9, 21, 23). A few small pigment spots may be observed in living larvae. Normal larvae move in place for awhile but very shortly become surface swimmers. They swim forward, at the same time spinning clockwise on the longitudinal axes when viewed from the animal pole.

By slowing down swimmers with MS-222 and observing them with the phase microscope (using dark-field which gives a strikingly beautiful picture), it is possible to get a "head-on" view of the former animal pole region. If the larvae spin slowly enough, one can see what looks like a diagrammatic representation of both the apical rosette (blastomeres $a_{1.3}$–$d_{1.3}$) and annelid cross (compare Plate XI, Figure 18, on Amphitrite in Mead's paper, 1897, page 311, or Figure 196-5, based on Nereis, in Borradaile and Potts, page 283). Blastomeres $a^{1.2}$–$d^{1.2}$ are less dis-





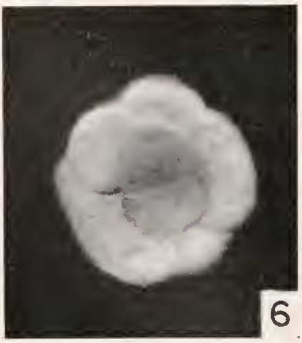


PLATE I

tinct but can be made out (blastomeres $a^{1.2}$–$d^{1.2}$ and $a_{1.3}$–$d_{1.3}$ are apparently designated as a^{12}–d^{12} and a^{13}–d^{13} in Borradaile and Potts). The four anterior plates of cells appear to arise from the four groups of prototroch cells and thus mark the position of the prototroch proper beneath them. The apical rosette forms the tip of the central mound of cells. Sometimes one or two globules (probably polar bodies which have not yet disintegrated) are seen in the space between the central mound and the membrane (Fig. 21).

The central mound in some 7-hour swimmers has grown almost to the animal region of the membrane. The apical tuft, in some larvae at least, appears one to two hours later. The cilia of the apical tuft have their origin from the central cells at the tip of the mound (in the few cases measured, cilia were approximately 40 micra when first formed). Their origin is not surprising, for, as noted above, the central cells make up the apical rosette which has been shown in other polychaetes to become the apical organ of the trochophore.

Continuous with the four anterior vacuolated plates, but extending posteriorly, are at least four yolk plates. Their formation leaves a space between them and the medial endodermal yolk mass of the larva. Anterior vacuolated plates and posterior yolk plates merge in the peripheral portion of the larval mass at about the equatorial level. Yolk spheres similar to those in these curious thin plates can be traced in serial sections from the posterior part of the larval mass peripherally and anteriorly (where some are observed at the base of the vacuolated plates) and then posteriorly just under the cuticle where they form thin plates. The yolk plates thus appear to arise from the posterior part of the larval mass (original vegetal hemisphere). The narrow spaces between each plate and the median mass are continuous with each other posteriorly and are visible as slits in some 12-hour larvae (and older) when they rotate. All normal larvae of 12 hours have a prominent apical tuft (one measured approximately 100 micra) which can be seen under the dissecting microscope. Larvae swim rapidly about the antero-posterior axis much as before with the apical tuft directed forward.

All figures are photomicrographs taken with a Makam camera. Figures 1 through 15 are all of living stages, taken at × 100, without a cover glass except for Figure 15. Moving stages were quieted with MS–222 (tricain). Figures 16 through 28 are all of sectioned material, taken at × 352 except for 27 and 28 which were taken at × 220. Figures 29 through 34 are all photomicrographs taken from "dry" mounts of larvae at × 430.

Plate I

Explanation of Figures

Figure 1. Developing eggs before their growth period is completed, showing algal-like strings of cells. Figure 2. Unfertilized egg showing lighter granular area at the animal pole where the polar bodies will pinch off. Figure 3. Fertilized egg with cleavage furrow beginning first at the animal pole. Figure 4. Two-cell stage, showing that the CD blastomere is somewhat larger than the AB. Note the fertilization membrane and one of the polar bodies. Figure 5. Four-cell stage viewed from animal pole, showing the cross furrow characteristic of spiral cleavage. Figure 6. Eight-cell stage in two tiers, four slightly smaller micromeres towards the animal pole. Figure 7. Early to mid-cleavage showing individual blastomeres. Figure 8. Early swimming stage, approximately four hours old, showing two of the four plates surrounding the central mound. Figure 9. "Head-on" view of stage similar to Figure 8, showing four plates of cells (one at lower right clear) surrounding the mound. Figure 10. Trochophore, approximately 28 hours old, with apical tuft and prototroch (haze at right represents the beating cilia).

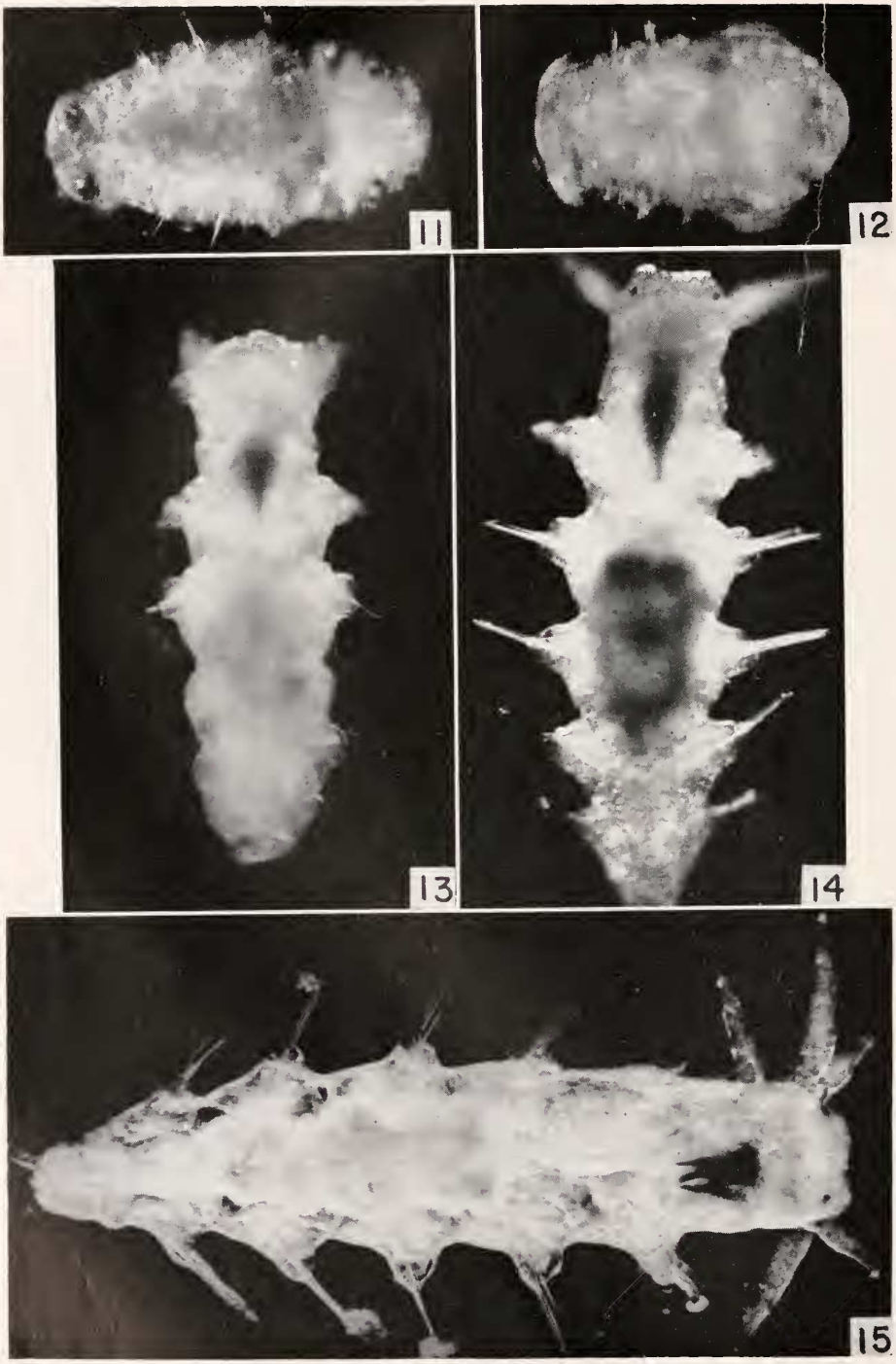


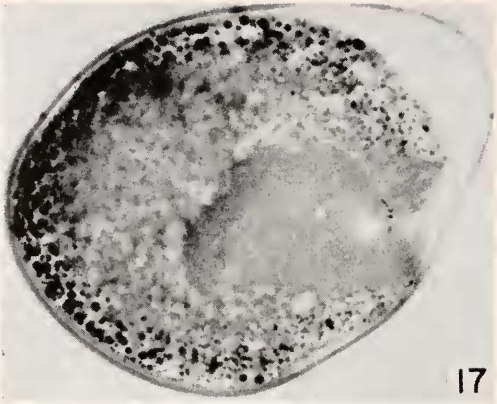
PLATE II

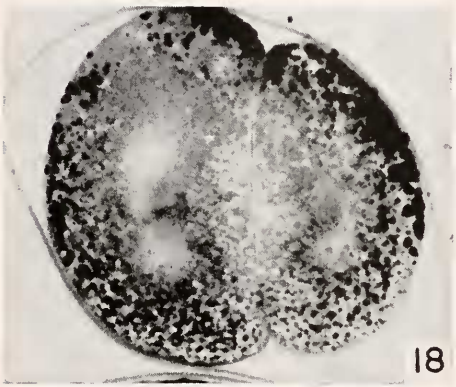
Trochophore stage, 24 hours old. Larvae of this stage are still approximately the size of the unfertilized egg and are positively phototactic active swimmers, rotating clockwise as in the preceding stages. Anteriorly, they have two red eyespots and a prominent apical tuft, 60–70 micra or more in length, consisting of several long cilia surrounded by a ring of shorter cilia. The body is still covered with cilia except for the small disc at the posterior end and a small area around the apical tuft (Fig. 10). The cilia appear somewhat longer in the region of the developing prototroch and telotroch. The denser central mass of cells represents the differentiating yolk-laden mid-gut (Fig. 22). In living stages a slight indentation observed on one side may represent the stomadeum. A few dark pigment spots (green in dark-field) in the region of the broad prototroch tend to mask the pharynx in living larvae. In some larvae the slit-like spaces formed between the posterior yolk plates and the underlying larval mass are still obvious; in others, growing cells have obliterated the slits so that the peripheral yolk plates are caught between the mesodermal bands and the cuticle. The yolk plates are then visible as a line of yolk spheres just beneath the cuticle (barely visible in Fig. 22). Thus, posteriorly, the layers from inside out are the central yolk mass, mesodermal bands, slits (in some instances), yolk plates, and larval membrane (Fig. 22).

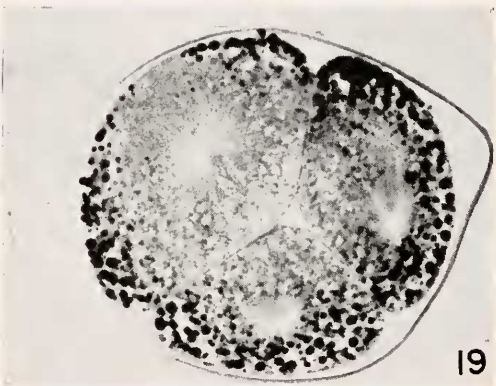
Post-trochophore stage, 36 hours old. This stage is usually little longer, though somewhat narrower, than the preceding and is characterized internally by the beginnings of two to three sets of setae and the formation of glandular cells (probably mucous in nature; Figs. 24, 25, 26). The larvae are strongly positively phototactic as evidenced by their swarming toward the light. They have prominent red eyespots and a well developed apical tuft (most of the cilia are approximately 85 micra, the longest measured being approximately 100 micra). The cytoplasm at the level of the broad prototroch has a bubbly appearance due to refractile droplets which tend to obscure the pharynx. Other external features are the narrow telotroch, short cilia between the proto- and telotrochs, and possibly a posterior tuft of cilia (a suggestion of this last was observed only twice, with the phase microscope). Yellowish pigment may be observed scattered over the surface. Visible under the cuticle posteriorly are the peripheral yolk plates. The gut from an external view is similar to that in the preceding stage, forming a darker central mass (yellow in reflected light). A posterior indentation may represent the proctodeum, as the hind-gut has not yet formed. The larvae appear to be flattened slightly on the ventral surface. Serial sections reveal the pharynx, differentiating setae, mucous cells, and four large posterior vacuoles which probably represent the free ends of mucus-secreting cells (Figs. 24 and 26).

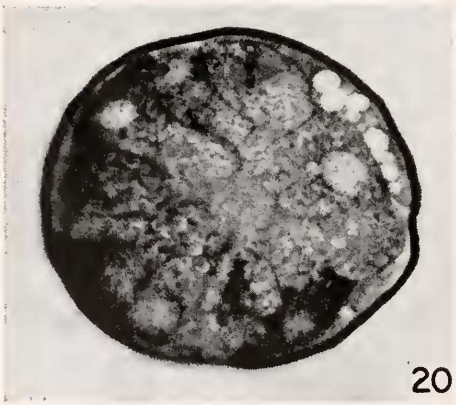
Plate II
Explanation of Figures

Figure 11. Larva of 3½ days with four sets of setae (the fourth set has retracted). Visible are two eyespots at right, two posterior vacuolated cells at left, and darker mid-gut region between the setae. Figure 12. Swimming larva, also 3½ days old, with the fourth set of setae just emerging. Note the beating prototroch at eye level and the telotroch at left. Figure 13. Larva of approximately four days, showing four sets of setae, three dorsal tentacles beginning to form, and black jaws visible through the body wall. Figure 14. Larva of six days with five sets of setae and dorsal tentacles elongating. Note the eyespots, dark jaws, and dark mid-gut region. Figure 15. Larva of seven days, with five sets of setae, photographed with cover glass. Visible are two eyes, "knobby" tentacles, black jaws, light mid-gut region, and two anal cirri.









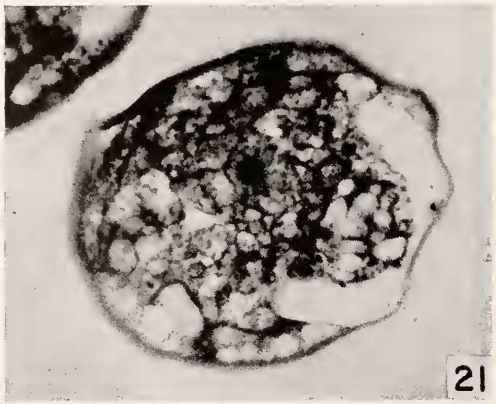


PLATE III

EXPLANATION OF FIGURES

FIGURE 16. Section of two young eggs in the coelom, showing attached algal-like strings of cells (second string not in plane of section), the nucleus and prominent nucleolus in the egg and in each of the "nurse" cells. FIGURE 17. Fertilized egg in metaphase I, showing the

Larvae of 2 days, 8–12 hours. These larvae, about the same width as the preceding, have elongated by about 100 micra and measure approximately 325 × 200 micra (in measurements of larvae, widths indicate the broadest portion). The tendency of some larvae to settle on the bottom at this stage seems to be correlated with the secretion of mucus; other larvae, however, are still actively rotating, positively phototactic swimmers. Their invariable swarming towards the light makes changing the water easy at this stage. The larvae usually have differentiated three sets of setae externally (sometimes only two), with a fourth set forming internally in some. The third set, though extending externally, may be incompletely formed (see Table I).

Larvae have two prominent red eyespots and several pigment spots anteriorly. The apical tuft, though reduced, is still prominent, being roughly 55 micra long. The anterior arms of the opaque Y-shaped mid-gut surround the colorless pharynx. Scattered black pigment spots can be seen in surface view. The prototroch is still present as is the telotroch of longer cilia, and between them are shorter cilia. Rarely seen, but very clear when observed with the phase microscope, is a little patch of cilia just posterior to each set of setae. The characteristic refractile droplets are still present at the widest part of the prototroch and this area appears continuous with the mid-gut region. The hind-gut is not clearly defined.

Larvae of 3 days, 8 to 12 hours. Larvae of this stage are slightly longer and usually somewhat narrower than those of the preceding stage (for example, one measured 400 × 180 micra). A few are still swimming and are positively phototactic, but most tend to crawl on the bottom, secreting mucus as they do so. They sometimes stick together in clumps in which case they should be separated before they die. Some have formed transparent slime tubes. Usually four functional sets of setae are visible externally (Figs. 11 and 12) and the parapodium of the first setigerous segment has two protrusions, a finger-like postsetal lobe and a shorter presetal lobe (Fig. 33). A tuft of cilia, rarely observed, is present at the base of each parapodium. An apical tuft is still prominent but is often missed even with the phase microscope, for it tends to bend backward when slowed with MS-222. The fairly broad prototroch extends from the anterior level of the eyespots to just anterior to the first set of setae (compare Figures 11 and 12). The prominent telotroch lies just posterior to the last set of setae (Fig. 12). Incipient jaws have differentiated which have an extra toothed plate on one side of the otherwise symmetrical maxillae (similar to Fig. 29). This asymmetry of the jaws is characteristic of the adult. These larval jaws are movable indicating that pharyngeal muscle is differentiating. Peripheral vacuolated mucous cells are clearly defined. Two of the large posterior vacuoles may be visible externally (Fig. 11). The broad anterior region, with its bubbly cytoplasm, still appears

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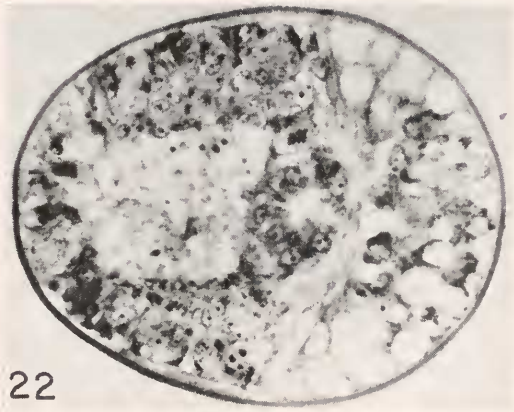
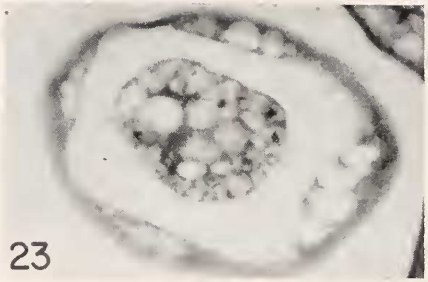
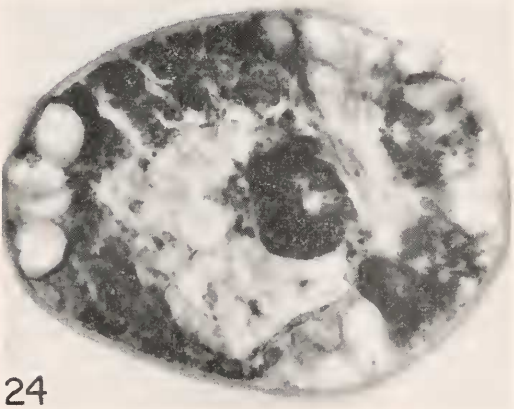
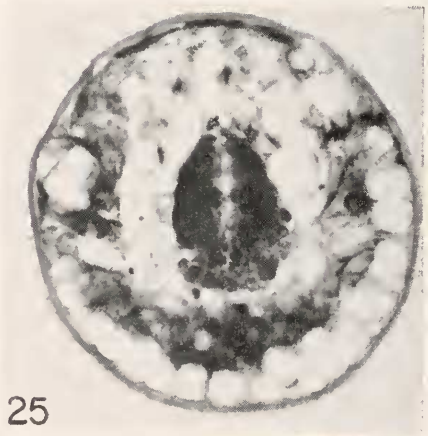
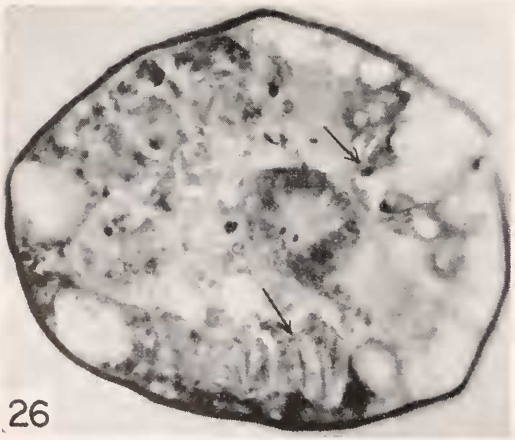
contrast between yolky and non-yolky cytoplasm. FIGURE 18. Two-cell stage in metaphase of second cleavage showing that the CD blastomere is larger than the AB. Note the fertilization membrane. FIGURE 19. Three-cell stage showing that the CD blastomere sometimes cleaves before the AB. This may represent abnormal development. FIGURE 20. Blastomeres of late cleavage held firmly within the egg membrane. The peripheral vacuolated region is beginning to appear and one blastomere is in metaphase. FIGURE 21. Longitudinal section through an early ciliated stage, approximately three hours, showing central mound of cells at the animal pole, two of the four vacuolated plates of cells, and small round body (probably a polar body) beneath membrane at the right.











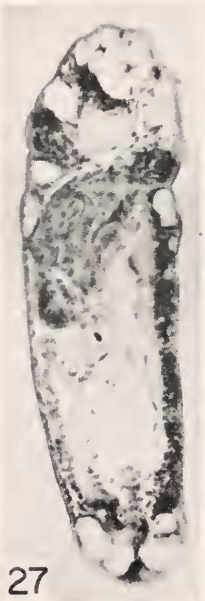


PLATE IV

continuous with the droplet-filled darker mid-gut region (Fig. 11, droplets not in focus). The arms of the Y-shaped mid-gut surround the pharynx. The thick-walled, rather transparent hind-gut, presumably ectodermal, is forming. In some batches, buds of the three more dorsal tentacles are obvious, as well as the rudiments of the two anal cirri.

Further internal structure can be seen in serial sections. Figure 27 is a sagittal section of this stage, showing pharynx and incipient jaws, narrow esophageal portion, and the mid-gut which has no lumen as yet and contains some dark pigment spots. A coelom has appeared, two flattened nuclei of the ventral peritoneal cells being clearly visible. The ventral body wall is thick compared with the dorsal and a ventral nerve cord is differentiating just beneath the peritoneum. A cerebral ganglion is visible just anterior to the pharynx. At least four large posterior vacuoles are visible.

Larvae of 4 days. By this stage four sets of setae are visible externally and a fifth is beginning to form internally. The apical tuft was not observed and proto- and telotrochs are reduced. A few superficial scattered dark pigment spots can be seen in living larvae, and the endodermal and mid-gut contains some pigment. The transparent hind-gut has a narrow lumen. In most larvae, three well developed tentacular protrusions have appeared (Fig. 13) and buds of the two more ventral tentacles, as well as two anal cirri. Also visible through the body wall are the developing jaws (Fig. 13).

Larvae of 4 days, 8 to 12 hours. Larvae of this stage have settled on the bottom and some may be observed in transparent slime tubes. They have four sets of functional setae externally with a fifth beginning to protrude in some. The presetal and postsetal lobes on the parapodia of the first setigerous segment are retained in this stage and in the subsequent stages described (compare Fig. 33). Five occipital tentacles are present, one mid-dorsal, two dorso-lateral, and two ventro-lateral ones, the last two being shorter. Two anal cirri are represented by

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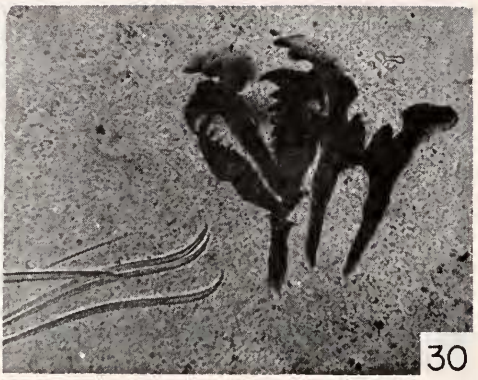
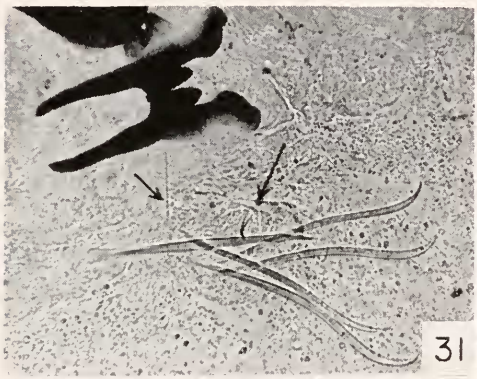
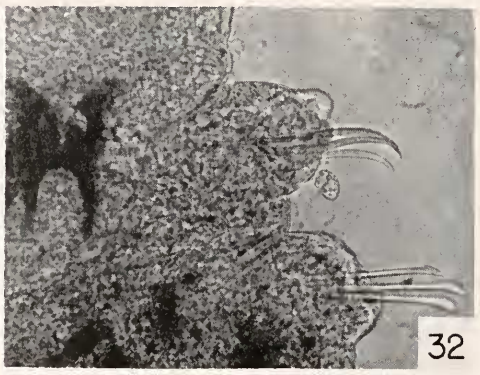
PLATE IV

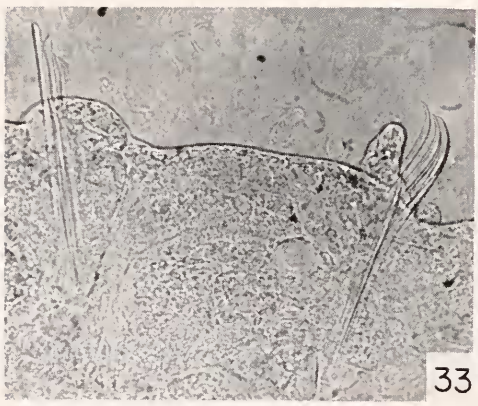
EXPLANATION OF FIGURES

FIGURE 22. Frontal section of 24-hour trochophore (anterior at right) showing pharynx near center, light undifferentiated yolk mass just posterior to it, and mesodermal bands flanking the mid-gut. FIGURE 23. Transverse section through the central mound in a larva similar to that in Figure 21, showing the four plates of vacuolated cells surrounding the mound. FIGURE 24. Frontal section through a 36-hour larva (cut at 15 micra) showing pharynx (note anaphase), light undifferentiated yolk mass, and four prominent posterior vacuoles. FIGURE 25. Transverse section through the pharynx of a larva that is similar to Figure 24, showing peripheral vacuolated cells and the cilia penetrating the larval membrane. FIGURE 26. Frontal section through a 36-hour larva (cut at 10 micra) showing the pharynx (note anaphase), yolk mass, and two large posterior vacuoles. Two sets of internal setae are forming (tip of lower arrow) and two of the mucus-secreting cells with basal nuclei are visible (tip of upper arrow). FIGURE 27. Sagittal section through larva of 3⅓ days, with four sets of setae. The jaws are beginning to form in the pharynx, the cerebral ganglion (light area) is anterior to them, and the mid-gut (without a lumen) is posterior to them. Note also the posterior vacuoles, the coelom around the gut, and the peritoneal cells (two nuclei clear) lying in contact with the ventral nerve cord. The ventral body wall is thicker than the dorsal. FIGURE 28. Sagittal section through larva of 5½ days, with five sets of setae. The same structures seen in Figure 27 may be noted, although they are more highly differentiated. The mid-gut region now has a lumen continuous with the intestine which opens by way of a ventral anus, and some of the mid-gut cells have black pigment.









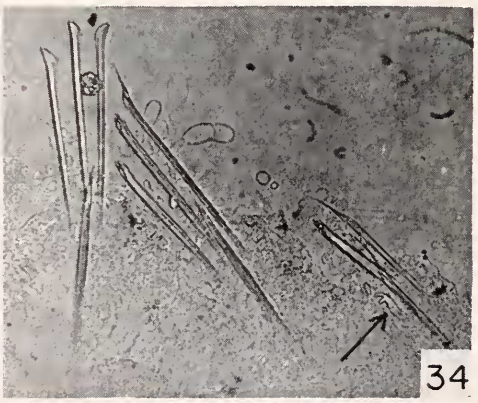


PLATE V

EXPLANATION OF FIGURES

FIGURE 29. Differentiating jaws of a larva of 4⅓ days showing toothed asymmetrical maxillary plates on the left (an extra toothed portion is present on the left side) and mandibles on the right. Note also the bundle of curved pointed setae from the first setigerous segment. FIGURE 30. Jaws from a larva of approximately eleven days, showing further differentiation

buds in some larvae of this stage, but are more obvious in others. Tufts of cilia, visible at the eye level in some, probably represent the remains of the prototroch. A prominent telotroch is still present. Also visible externally are jaws consisting of asymmetrical maxillary plates with well defined teeth and differentiating mandibles (Fig. 29). An esophagus is differentiating between pharynx and mid-gut, and the latter continues posteriorly into the hind-gut. The dark yolk mass and droplets are restricted to the mid-gut and black pigment is visible in its lining. Some of the larvae appeared to be feeding on microorganisms.

Larvae of 5½ to 7½ days. Larvae of 5½ days have 5 sets of functional setae although the last set is usually not completely formed; in some cases a sixth set is differentiating internally. Some larvae may be observed in transparent slime tubes on the bottom, and in one instance a larva was observed turning around in its tube. Larvae which have not formed tubes often stick to the bottom at this stage and may constrict in two in attempting to free themselves. The five occipital tentacles are "knobby" and well developed (Fig. 15): the three more dorsal ones are approximately 150 micra in length and have two basal segments by 7 days; the two more ventral ones are shorter and have one basal segment each. Two anal cirri are well developed (approximately 30 micra in length) and "knobby" (Fig. 15).

A number of the differentiating internal structures of this stage can be illustrated by Figure 28. This is a sagittal section through a larva with 5 sets of setae (5½ days old) and with well developed jaws associated with the pharynx. The mid-gut is patent throughout, its lumen being continuous with that of the hind-gut which, in turn, opens ventrally through the anus. The coelom has enlarged as compared with the preceding stage (Fig. 27). Nuclei of two of the flattened peritoneal cells are visible ventrally (the peritoneum can also be seen in living larvae), and the cerebral ganglion and ventral nerve cord are clearly visible.

Larvae of 8 days, 8 hours and older. By 8⅓ days, 6 sets of setae have formed externally in most cases and are complete, or almost so. However, some larvae take one to three days longer to form the sixth set (a few take even longer). The black jaws are well differentiated and active at these stages. The asymmetrical maxillary plates have a medial toothed margin in each half (as well as the toothed

as compared with Figure 29. The bundle of curved pointed setae from the first setigerous segment and an additional slender rod are also visible. FIGURE 31. Curved pointed setae on the first setigerous segment of a larva of 8½ days, with six sets of setae. Characteristically, four such setae are present but here the curved tip of a fifth set is appearing (off tip of right-hand arrow). Note also the aciculum with a deeper origin than the external setae, and the slender rod (off tip of left-hand arrow). FIGURE 32. Two anterior parapodia in a larva of approximately 5 days, with four sets of setae. The curved, pointed, claw-like setae of the first setigerous segment are visible; contrast these with the short-tipped winged capillary type (one in focus) characteristic of the second, third, and fourth setigerous segments. FIGURE 33. Parapodia of first and second setigerous segments (anterior at right) in a larva of 5⅓ days, with a small fifth set of setae. The finger-like postsetal lobe and the smaller presetal lobe which are characteristic of the first parapodium are visible. FIGURE 34. Setal types from the fourth, fifth, and sixth setigerous segments (anterior at left). Note the three short-tipped winged capillary setae (and basal aciculum) characteristic of the second, third, and fourth setigerous segments, the two bidentate acicular setae and one long-tipped winged capillary seta (and basal aciculum) characteristic of the fifth, sixth, and seventh setigerous segments. The two-pronged tip (off tip of arrow) of the second bidentate acicular seta developing in the sixth setigerous segment is also visible.

additional piece; see Figure 30) and work in scissors-like fashion with the mandibles either held stationary or with both jaws working alternately in an antero-posterior direction. The maxillary plates move forward, open, and then close during their posterior movement.

In a few cases a culture of algae was allowed to accumulate in the stender dishes. The larvae in these cases appeared to be feeding on the algae although the mid-gut was still dark with stored food material and contained large food vacuoles. The larvae upon occasion will eat their own kind as in one instance black jaws of another larva were observed in the mid-gut of an 11½-day larva, and one larva appeared to be "gnawing" on another living larva stuck to it. An active rolling movement from side to side was noted in the esophageal region of a number of larvae, and in one food particles were noted in this region of the fore-gut which is very thick-walled.

The five occipital tentacles are similar to those of the preceding stage except that they are longer, the dorsal ones measuring approximately 225 micra in 9-day larvae. Anal cirri in larvae of this age are approximately 50 micra long.

Headless larvae, capable of moving about, were observed occasionally. Larvae of this age tend to stick to the bottom of the dish, often on their backs, in which case they may constrict in two in an attempt to become free.

The larvae were not fed (except for any microorganisms which came through the sand-filtered sea water) and may live as long as the yolk material lasts in the mid-gut (this area becomes transparent when the food supply is gone). Over several summers, 6 sets was the maximum number of setae observed in these larvae of *D. cuprea*. However, in the summer of 1958, 7 sets were recorded for nine larvae, in two (from different batches) by 13½ days of development, in one by 14½ days, in two (from different batches) by 17½ days, and in one by 18½ days of development. One larva from this last batch did not develop a seventh set until the twenty-fifth day, and another from this batch until the thirtieth day of development. One from a different batch developed a seventh set by the twenty-sixth day. Among these larvae the oldest lived for 13 days after developing a seventh set of setae, dying at an age of 30½ days. Most larvae died before developing a seventh set. The types of setae are described in more detail below.

Types of larval setae and their order of appearance. By the time 5 sets of setae have formed in these larvae, four types of setae have differentiated. The type (or types) and distribution of each are characteristic for each segment. As indicated in Figures 29 to 34, those in the first setigerous segment are different from any of the others, those in segments two, three and four are similar, and those in segment five are new types which are retained in segments six and seven. One aciculum is associated with each setigerous sac at all levels. These internal basal setae have a deeper origin than the others (Figs. 31 and 34) and appear to direct the movements of the external ones. Once the direction of movement has been determined at any one level, the external setal complement seems to work against the aciculum which thus acts as a fulcrum.

The following tables indicate the setigerous segments, the number and types of setae in each setigerous sac (omitting acicula which are present at all levels), the time of appearance at each level, and the setal complement of each segment at successive developmental stages. Photomicrographs are presented to help in the

TABLE I

Time of appearance of setal types in various segments

Setigerous segment	Type of setae	Time of external appearance
1	3C	2 days
	3C + tip of C	$3\frac{1}{2}$ days
	4C	$4\frac{1}{3}$ days
2	2S	2 days
	3S	$2\frac{1}{2}$ days
3	2S	$2\frac{1}{2}$ days
	3S	$3\frac{1}{2}$ days
4	2S	$2\frac{1}{2}$ days
	3S	$3\frac{1}{2}$ days
5	1B, 1L	$4\frac{1}{2}$ days
	2B, 1L	$5\frac{1}{2}$ days
6	1B, 1L	7 days
	2B, 1L	$8\frac{1}{2}$ days
7	1B, 1L	13 days (typical?)
	2B, 1L	?

identification of these setal types. The key to the letters in the tables is as follows: C—curved pointed type (Figs. 29 to 33), S—short-tipped winged capillary type (Figs. 32 to 34), B—bidentate acicular type (Fig. 34), L—long-tipped winged capillary type (Fig. 34).

The individual setae develop in a disto-proximal direction, the tip differentiating first. This was observed repeatedly in "dry" mounts. For example, in the first setigerous segment of a 4-day larva, three curved setae are complete and just the curved tip of the fourth is visible internally. In the fifth setigerous segment of 4- to 6-day larvae, one of the bidentate setae and the aciculum appear to develop simultaneously; then the long-tipped seta of this level develops and before it is completed the two-pronged tip of the second bidentate seta has developed internally (Fig. 34). This sequence of setal development noted in setigerous segment number five is followed also in the sixth and seventh segments.

In one larva ($8\frac{1}{2}$ days old) the distal tip of a fifth seta of the curved type characteristic of segment 1 was noted (Fig. 31). This indicates that 4 curved setae may not be the full complement for this level; however, this one case may not represent the typical condition. Also, in a number of larvae of 8 days, 8 hours

TABLE II

Distribution of setal types by segments at different stages

Larval stage	Setigerous segment						
	1	2	3	4	5	6	7
3 parapodia	3C	3S	3S				
4 parapodia	3C	3S	3S	3S			
5 parapodia	4C	3S	3S	3S	2B, 1L		
6 parapodia	4C	3S	3S	3S	2B, 1L	2B, 1L	
7 parapodia	4C	3S	3S	3S	2B, 1L	2B, 1L	2B, 1L

and older, a tiny slender rod was noted in both of the first setigerous sacs (Figs. 30 and 31). Its presence was not observed consistently throughout this age group.

As suggested by the tables, the setae once formed were retained throughout the period of observation. This is in contrast to Wilson's analysis of the succession of larval bristles in *Nereis pelagica* (1932) in which he found that as successive setae formed, the ones more anterior began falling out.

Discussion

Certain aspects of the development of the egg and of the early larvae of *Diopatra cuprea* seem to be peculiar to this species, and in other instances to this genus or to the closely related genus, Onuphis. The curious process by which the eggs are formed in the ovary has been described by Andrews (1891b) and recently has been briefly reviewed by Costello *et al.* (1957). Lieber (1931) has described this process for *D. amboinensis*. Andrews (1891b) suggests that the algal-like strings of "nurse" cells attached to the developing egg may have a supportive function while the eggs are floating free in the coelom, rather than a nutritive one. However, Treadwell (1921, page 81) states that in the eggs of *Diopatra cuprea* at Woods Hole he was able to demonstrate a "definite communication pore between the ovum and the first cell of the chain, indicating that they are true 'nurse' cells." Lieber (1931) in a detailed study of oogenesis in Diopatra described and figured a cytoplasmic connection between the developing egg of *D. amboinensis* and its attached "nurse" cell and concluded that the cells were, in fact, nutritive in function and, therefore, properly termed nurse cells. The communication pore noted by Treadwell (1921) may conceivably represent the area where an amoeboid process of the egg could contact the cytoplasm of the "nurse" cell.

Lieber (1931) has described a micropyle in the egg membrane of *D. amboinensis*. The defect observed near the vegetal pole in some eggs of *D. cuprea* in the present investigation may be a micropyle, although Andrews (1891b) makes no mention of it in either *D. cuprea* or *D. magna*. These defects may instead represent the remains of the communication pore noted by Treadwell (1921) in the developing oocyte.

It has been noted that the ripe eggs of *Diopatra cuprea* appear to be perforated. The canalicular nature of the membrane has been demonstrated in stained eggs of Diopatra by Lieber (1931). A porous membrane is not restricted to the eggs of Diopatra but has been noted in other polychaete eggs, for example, those of *Arenicola cristata* (Wilson, 1882).

Retention of the egg membrane as a larval cuticle (noted in *D. cuprea*) apparently is not uncommon among polychaetes. Wilson (1882, page 295) states, "The persistence in some cases of the chorion as the larval cuticle is a remarkable occurrence entirely confined, so far as known, to the Chaetopods and Gephyrea, and by no means universal among them." Examples of species which retain the original egg membrane are *Clymenella torquata* and *Arenicola cristata* (Wilson, 1882), *Nereis diversicolor* (Dales, 1950), and *Tharyx marioni* (Dales, 1951).

The four anterior vacuolated plates of cells which have formed by the time ciliation has been attained are peculiar to this form insofar as the writer knows, and appear to originate from the four groups of prototroch cells.

The significance of the curious arrangement of yolk spheres into peripherally located yolk plates has not been determined, for the main mass of yolk remains in the central endodermal position (mid-gut region) of the trochophore. One possibility is that these peripheral plates may serve as a more efficiently placed food supply for the rather precocious development of the setae and associated muscle strands which differentiate from the mesoderm just medial to them.

As has been noted in the introduction there seems to be considerable confusion in the literature concerning the identification of larvae and earlier stages ascribed to *Diopatra cuprea*. It is well known that larval types are difficult to identify. Two important characteristics used for distinguishing between larvae are the jaws and setal types. The conspicuous asymmetry of the maxillary plates in *Diopatra cuprea* has been noted (Figs. 29 and 30). Monro (1924), in his description of the post-larval stage of *D. cuprea*, also pictures the unpaired, toothed plate associated with the otherwise symmetrical maxillae. This asymmetrical jaw type is characteristic of adult onuphids and eunicids. The functional significance of unpaired maxillary plates in otherwise symmetrical jaws, which appear to work in scissors-like fashion, is obscure. Comparing the diagram of the upper jaw pictured in Monro (1924, Fig. 6, page 197) with the writer's photomicrograph of the jaws of an 11-day larva (Fig. 30), one may conclude that they are closely similar and in all probability could have come from larvae of the same species when one considers the difference in age. Monro (1924) includes a brief discussion of the possible evolution of jaws within the eunicids and closely related groups.

Setae develop precociously in *Diopatra cuprea*, at least as compared with some of the nereids, such as *Nereis pelagica* (Wilson, 1932) and *Nereis diversicolor* (Dales, 1950). The importance of setal types in distinguishing between larvae is indicated by the work of Wilson (1932), Krishnan (1936), Dales (1950), *et al.* A comparison of the setae pictured here with the description and diagrams in Monro's post-larval stage (1924) suggests that the larvae described by Monro belong to a closely related species, if not to *D. cuprea*. Development of the first setigerous segment (Monro, 1924, Figure 2, and text, page 195) is in agreement with the findings described in the present study, but Monro indicates that from the second through the fifth set all setae are of the short-tipped winged capillary type. The view pictured is not clear (Fig. 3, page 195), and this setal type may or may not fit the type shown in the present investigation (Figs. 32, 33, and 34). In contrast to Monro's larvae, the fifth set of setae observed in the present study has a new setal complement which includes a bidentate acicular type which is retained in segments 6 and 7 (Fig. 34). Beginning on the sixth segment of Monro's larvae a setigerous type (Fig. 4, page 196) appears which probably could be developed from the bidentate acicular type described here (Fig. 34) by the development of a hook. However, to be comparable to the larvae described by the writer, this hooked type should begin on the fifth parapodium instead of the sixth. Thus, the two species may not be identical.

Wilson (1882) describes and figures some early stages in the development of a polychaete which he identifies as *Diopatra cuprea*. These larvae, however, were obtained from gelatinous egg masses, and Andrews (1891a, 1891b) states that these early stages and larvae described by Wilson do not belong to *Diopatra cuprea* but to *Diopatra magna*. Monro (1924) notes that Andrews does not give the

basis for his statement and Monro, therefore, questions its validity. Treadwell (1921) has shown that the polychaetes described in the literature as *D. magna* in reality belong to another genus which he has designated as Onuphis. Both Diopatra and Onuphis are now accepted as distinct genera although they are closely related ones (Dr. Marian H. Pettibone, personal communication; also see Hartman, 1945, page 24, and Hartman, 1951, page 51, for keys separating these two genera). Treadwell (1921) further points out the possibility that the larvae described by Wilson are really those of *Onuphis magna* and seems inclined to agree with Andrew's interpretation. A comparison of the ciliated larva pictured by Treadwell from the gelatinous egg masses of *Onuphis magna* (1921, Plate 7, Figure 5) with that figured by Wilson (1882, Plate XXIII, Fig. 10) shows more similarity between these two larvae than between Wilson's larvae and those of *D. cuprea* described in the present study.

Comparing Wilson's larvae with the larvae pictured here, raised from the fertilized eggs of *D. cuprea*, certain differences are noted. No stages in the present study were observed that were as pear-shaped as Wilson's Figures 89 and 90 (Plate XXI), nor was any stage observed so markedly spotted with pigment as the larva in Wilson's Figure 89. Further, the rudimentary apical tuft shown is in marked contrast to the prominent apical tuft in the larvae here described. A comparison of larvae with five sets of setae shows that there are differences between those of Wilson (1882, Plate XXIII, Fig. 10, and description on page 289) and those pictured and described by the writer. In *Diopatra cuprea*, in the present study, no dorsal cirri were observed, five occipital tentacles are present in normal larvae at this setal stage, and the mid-dorsal tentacle is almost the same size as the dorso-lateral (contrast Wilson's Fig. 10, Plate XXIII). Also a clearly defined pharynx and well developed jaws are visible at this stage (Figs. 14 and 15 of the present paper; however, Wilson and Treadwell may have intentionally omitted internal structures from their drawings). Further, the enlarged tip of the one setal type shown in Wilson's larva (Plate XXI, Fig. 91) is different from any here described for *D. cuprea* (Figs. 31 and 34), although it is possible that this type might develop in a later stage.

Distribution of the two species in question provides further evidence concerning the possibility of erroneous identification of their larvae. Both *Diopatra cuprea* and *Onuphis magna* are found intertidally in the Beaufort, North Carolina, area (Hartman, 1945) and in the Gulf of Mexico (Hartman, 1951); there is, therefore, a chance of confusing the egg cases of the two genera in these areas. Thus far, however, *D. cuprea* is the only onuphid found intertidally in the Woods Hole area (Dr. Marian H. Pettibone, personal communication), so to date there is no possibility of confusion between these two onuphids (*D. cuprea* and *O. magna*) in the intertidal zone at Woods Hole. The writer is led to the conclusion, therefore, that the stages pictured by Wilson do not belong to *Diopatra cuprea* and probably belong to *Onuphis magna* (*D. magna* of Andrews) as Andrews has stated.

If Andrews is correct—and the evidence presented here indicates that he is —then the gelatinous egg masses found by Wilson belong to *Onuphis magna*. Insofar as the writer knows, gelatinous egg masses of *D. cuprea* have never been found in the Woods Hole area where this species is common. She herself has never observed them and Mr. Milton B. Gray, who has collected *D. cuprea* for

a number of summers in the Woods Hole area (both for investigators and for Course work), has never seen them (personal communication). Circumstantial evidence presented by Monro (1924) indicates that the eggs of *D. cuprea* are laid inside the tube (where the larvae develop) rather than in gelatinous egg capsules lying free on the sand. However, the possibility remains that Monro is not dealing with *D. cuprea* but with a closely related species. The one time normal spawn jelly was observed in the present study, it dissolved readily in sea water. This property of the jelly and the facts that cilia develop early and that the larva forms a prominent apical tuft suggest that *D. cuprea* may have a free-swimming stage.

The writer, with the above observations in mind, would like to suggest that the egg masses with developing larvae which have been noted along the Gulf of Mexico (Hartman, 1951) as well as at Beaufort, North Carolina (Andrews, 1891b; Hartman, 1945; Wilson, 1882), belong to *Onuphis magna* and not to *Diopatra cuprea*. Both species have been described as occurring together in these areas although their distribution along the Gulf of Mexico is somewhat different (Hartman, 1951).

With the confusion of these larval types apparent in the literature, the brief study of the setal types of *D. cuprea* included here may serve as at least one criterion for distinguishing between the species of onuphids in the future. The usefulness of setal types is apparent if one compares the table given by Krishnan (1936, page 521) for *D. variabilis* (Southern) with the tables included here for *D. cuprea*.

In summary, one is led to the conclusion that the early stages and larvae described by the several investigators cited probably do not belong to the species, *Diopatra cuprea*, but to a closely related genus or species, in two instances probably to *Onuphis magna* which is the *Diopatra magna* of Andrews.

Further, this would seem to indicate that the descriptions of the writer for *Diopatra cuprea* are the only ones which can be correctly attributed to this species, with the possible exception of Monro's post-larval description which may belong to *D. cuprea*. The possibility remains, however, that some investigation not here cited has escaped the writer's attention.

The problem of activation of the egg of *D. cuprea* will have to be solved before this egg can be used to any extent either for experimental purposes or for class use. Some histochemical tests have been run on these stages (Allen, 1957) and it is hoped that in working further with the eggs of *D. cuprea* some of the problems noted will be solved. Further details of development may then be worked out to serve as a basis for experimental and histochemical studies.

Summary

1. Larvae of *Diopatra cuprea* (Bosc) have been raised, following artificial fertilization, to a stage with seven sets of setae. Observations on living stages and also on fixed and stained preparations have been described and photographed.

2. Cell lineage studies have not been made, but observations indicate that the early cleavages are typical of those for spiral cleavage and that the ciliated stage (age, three hours) has a typical annelid cross and apical rosette. It, therefore, seems justifiable to conclude that the development of *Diopatra cuprea* follows the typical spiral pattern and mosaic development characteristic of other polychaetous annelids.

3. Peculiarities of the development of this polychaete, and possibly of closely related species, are the following: the peculiar algal-like nurse cells attached to the developing oocyte (also characteristic of Onuphis eggs) when floating free in the coelom, the amazing rapidity of development to the free-swimming stage (three hours), the four plates of cells which appear to develop from cells of the prototroch and their peculiar posterior extensions into at least four plates of yolk spheres, and the asymmetry of the maxillary plates.

4. Very little can be found in the literature on the embryology of the genus, Diopatra, and at least two authors have pointed out the possibility of error as to species in the identification of the developmental stages. Evidence is presented here which indicates that the early embryological and larval stages described by other investigators have been erroneously assigned to *Diopatra cuprea*.

5. If the above is correct—and it would appear that *Diopatra cuprea* is the only onuphid found intertidally in the Woods Hole area—one may conclude that the investigation presented by the writer is probably the only study recorded in the literature on the early developmental stages of *Diopatra cuprea* (Bosc). This is exclusive of Monro's description of the later (post-larval) stage which, if not belonging to *D. cuprea*, is undoubtedly closely related to this species.

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